(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 495 930 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
28.04.1999 Bulletin 1999/17

(21) Application number: **91905729.9**

(22) Date of filing: **28.01.1991**

(51) Int Cl.[6]: **G02B 21/00**

(86) International application number:
PCT/US91/00607

(87) International publication number:
WO 92/02839 (20.02.1992 Gazette 1992/05)

(54) Multicolor confocal fluorescence microscopy system

Konfokales Mikroskopsystem für Mehrfarbenfluoreszenz

Système à fluoréscence polychrome pour microscope confocal

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB IT LI LU NL SE

(30) Priority: **10.08.1990 US 565625**

(43) Date of publication of application:
29.07.1992 Bulletin 1992/31

(73) Proprietor: **REGENTS OF THE UNIVERSITY OF MINNESOTA**
Minneapolis, Minnesota 55455 (US)

(72) Inventors:
- **BRELJE, Todd, Clark**
  Minneapolis, MN 55414 (US)
- **SORENSON, Robert, L.**
  Minneapolis, MN 55417 (US)

(74) Representative: **VOSSIUS & PARTNER**
Postfach 86 07 67
81634 München (DE)

(56) References cited:
EP-A- 0 270 251
EP-A- 0 363 931

Printed by Jouve, 75001 PARIS (FR)

EP 0 495 930 B1

Description

Field of the Invention

[0001] The present invention relates to confocal microscopy and in particular to laser scanning confocal microscopy in which laser light is used to excite dyes in a specimen.

Background of the Invention

[0002] Confocal microscopy is well known in the art. The concept of confocal microscopy is that the image viewed by the microscope is confined to a very precise focal plane by limiting the depth of field of the image. Only those portions of the specimen which are in focus are detected. Out of focus regions of the sample appear dark. By changing the position of the focal plane, this important principle defines one major method for achieving optical sectioning.

[0003] The earliest versions of confocal microscopes used direct vision design with incoherent illumination. The field of illumination in the specimen was limited by a pin hole positioned on the illumination axis. The image of this pin hole is then projected on the specimen by a condenser lens. The illuminated point on the specimen reflects light (or as described below, emits fluorescent light). The reflected light of the image is then focused through an objective lens onto a detector. Either the specimen or the light focused on the image is scanned in a raster pattern so that the detector collects pixel information from a region of the specimen. The pixel information is then passed through a computer which can generate an image of the overall specimen.

[0004] The concept of the scanning confocal microscope is described in US Patent 3,013,467 to Marvin Minsky, which is hereby incorporated by reference. The optical path of the scanning confocal microscope may be constructed in trans-illuminating mode in which a separate condenser and objective lens is used in the same axis. In the alternative, the optical path of the scanning confocal microscope may be constructed in an epi-illuminating mode making a single objective lens serve both as the condenser and objective lens and using a dichroic or half mirror to collect the emitted light into a detector, as shown in Figure 1.

[0005] In the Minsky patent, the raster scan is generated by moving the stage on which the specimen is supported by two orthogonally vibrating tuning forks that are driven by electromagnets. As the stage is moved in a raster scan pattern, the resulting image detected by the image detector is a serial raster scanned image.

[0006] The use of fluorescent dyes to stain the specimen being viewed further improved the range of applications to which scanning confocal microscopy could be applied. Especially in the area of immunofluorescence histochemistry and in other neuroanatomical techniques, the staining of specimens with dyes is particularly useful to aid in distinguishing different features within biological tissues. The stains may comprise dyes designed only to absorb light or dyes that emit light in response to absorption, which is called fluorescence. Fluorescent dyes have the advantage over dyes which only absorb light in that a given fluorescent marker will be visible only when illuminated with the appropriate filter set.

[0007] Fluorescence is a consequence of the interaction of a photon with a fluorophore. When a photon of light is absorbed by a molecule it may increase the potential energy of the molecule by raising an electron to a higher orbital state. An electron raised to a higher orbital state from its natural state will tend to revert to the natural state. When the electron falls from a higher to a lower orbital state, energy is released which is equal to the difference in energy between the two orbital states. When this occurs, part or all of its energy may be released as a photon having a wavelength (spectral line) proportional to the released energy. The resulting luminescence is called fluorescence (and in some circumstances phosphorescence). Excitation of a fluorophore molecule at one wavelength typically results in fluorescent emission at longer wavelengths of light.

[0008] The scanning laser confocal microscope improved on the design of the scanning confocal microscope and the use of fluorophores by using coherent light to scan the stained specimen. The monochromaticity, high intensity and lack of divergence of the laser light contributed to improvements in the resulting images. In an epi-illuminating laser scanning confocal microscope of the prior art, as shown in Figure 2, the laser light 200 is scanned onto the specimen 220 from above and is reflected to a detector 215 in the same focal path as the incident light through the use of a half mirror or dichroic mirror 205. Typically, the specimen is stained with fluorescent dyes to enhance specific features within the specimen which may be of interest.

[0009] The MRC-600 laser scanning confocal imaging system, shown diagrammatically in Figure 2, is manufactured by BioRad Microscience of Hemel Hempstead, Herts, England. This laser scanning confocal microscope system is adaptable for use with a number of upright and inverted microscopes available from microscope vendors such as Nikon, Zeiss, Olympus and Leitz. The coherent illumination is an argon ion laser 201 having primary lines at 514 and 488 nanometers (nm). The emitted laser light 200 also has a plurality of minor spectral lines as determined by argon. The lines are filtered by the external filter 203 which selects either the 488nm (blue) line or the 514nm (green) line by means of an excitation filter 203. The selected light is reflected by a beam splitter 205 which includes a dichroic mirror

used for fluorescent imaging. If simple reflection imaging is required, a semi-reflecting or half mirror may be used in place of the dichroic mirror 205

[0010] The argon ion laser is available from Ion Laser Technology Company of Salt Lake City, Utah, part number 5425A. The argon ion laser is used to excite fluorescent dyes in the specimen which emit light slightly shifted in the spectrum in response to the excitation wavelength of the spectral lines of the laser light. The dyes are selected based upon their sensitivity to light, their affinity for features desired to be viewed in specimens and their fluorescent capabilities.

[0011] The light 200 from the laser is passed through the scanning unit 207 where it is raster scanned in an XY scanning movement by means of two oscillating mirrors. The laser beam is then passed through a microscope eyepiece onto the specimen such that a scanning spot caused by the scanning unit 207 scans the specimen. Reflected light or fluorescent light from the specimen passes back through the scanning system along the same path as the incident laser light. Reflection of the light is so rapid that the mirrors have not shifted position so that the light retraces the exact original path in the reverse direction. A portion of the reflected or fluorescent light passes through a half mirror or dichroic mirror 205 to be passed to photomultiplier tubes.

[0012] The laser scanning confocal imaging system from BioRad shown in Figure 2 attempts simultaneous imaging of two different fluorescent stains. The 514nm spectral line from argon ion laser 201 is used to excite both fluorescein isothiocyanate (fluorescein) and Texas Red™ (from Molecular Probes, Inc.) conjugated probes. This attempts the simultaneous excitation of different fluorescent dyes to allow selected features of the specimen to be stained in different colors and viewed together. The dual images are picked up by photomultiplier tubes 213 and 215. A second beam splitter 207 is a dichroic mirror allowing light of one wavelength to be directed to photomultiplier tube 213 while light having other wavelengths passed to photomultiplier 215.

[0013] The two images received from photomultiplier tubes 213 and 215 are used by a computer 222 to construct an image on display 224 of the specimen in a single focal plane. The simultaneous imaging of two different fluorescent stains at exactly the same focal plane would allow the identification of different specific features in the same specimen. A shortcoming of the dual color laser scanning confocal microscope system of the prior art is that the 514nm line of the argon ion laser produces simultaneous excitation of the two fluorescent dyes (fluorescein and Texas Red™). This simultaneous excitation causes false imaging and the loss of feature detail in the resulting image generated by the computer.

[0014] Figure 3 shows a graph published by BioRad Microscience indicating the absorption and emission spectra of fluorescein and Texas Red™. The graph is reproduced from BioRad and only approximates the spectrums. Curve 301 describes the absorption spectra of fluorescein while curve 303 shows the emission spectra of fluorescein. Curve 305 shows the absorption spectra of Texas Red™ and curve 307 describes the emission spectra of Texas Red™. As can be seen in Figure 3, there is an area of overlap between the absorption spectra of Texas Red" and fluorescein at 514nm. Thus, simultaneous excitation and emission of fluorescein and Texas Red™ occurs when excited with the single 514nm line of the argon laser. Also shown in Figure 3 is a large area of overlap between the emission spectra of Texas Red™ and the emission spectra of fluorescein.

[0015] The response curves for the filters and the dichroic reflectors are placed below the absorption and emission spectra of fluorescein and Texas Red™ in Figure 3 for comparison. When using an argon laser to excite the dyes, the 514nm line of the dye is the only line allowed to pass through the exciter filter 203 shown in Figure 2. The narrow wavelength response curve 309 of Figure 3 is for the exciter filter 203. The response curve 311 is for dichroic reflector 205 and the response curve 313 is for dichroic reflector 207. The response curve 315 is for green channel filter 211 and the response curve 317 is for red channel filter 209.

[0016] As can be seen in Figures 3, the intent is to have the single 514nm line of the argon laser excite both the fluorescein and Texas Red™ dyes. The emission spectra of these respective dyes are then selected to be passed to photomultiplier tubes 213 and 215 shown in Figure 2 to be independently detected for reconstructing a two color image at the same focal plane. The problem with this prior art technique is that the single excitation line from the argon laser excites fluorescein much more efficiently than Texas Red. For example, as shown in Figure 3, the excitation of the fluorescein dye at a wavelength of 514nm is at approximately 50%. The excitation of Texas Red™ at the same 514nm wavelength, however, is very low (less than 3%). Since the emission spectra of the dyes correspond to, and are proportional to the amount of energy absorbed by the dyes, the low amount of absorbed energy from the 514nm line by Texas Red™ will result in a very low amount of emitted fluorescent light. Hence, the amount of fluorescein emission seen in the red channel can vary according to the relative concentrations of fluorescein and Texas Red™. Unless the relative concentrations and saturation of the dyes accurately controlled, the emission spectra of Texas Red™ may be swamped by the "spillover" of the longer wavelengths of the fluorescein emission spectra. This confusion will result in images in which many of the features stained only with fluorescein will appear in both images. One solution to this problem is to use separate laser lines to better excite both fluorescent dyes.

[0017] Multiple line excitation of specimens dyed with different fluorochromes using two lasers is also known in the prior art. For example, a Spectra-Physics 2025 argon ion 3-watt water cooled laser (tunable to a single argon ion line

between 351nm through 526nm) has been confocally aligned with a 5 milliwatt air-cooled argon ion laser having fixed wavelengths at 488nm and 514nm. The alignment of two lasers. however, presents extreme focusing problems. The two light paths must be aligned to exacting standards to ensure that the same focal plane is observed.

Summary of the Invention

[0018] The shortcomings of the prior art described above and other shortcomings of the prior art which will be recognized and understood by those skilled in the art upon reading and understanding the present specification are overcome by the present invention. The present invention teaches a true multi-color laser scanning confocal imaging system for use with a microscope according to claim 1 in which a single laser having a multi-line output is used to simultaneously or individually excite a plurality of dyes. The images may be simultaneously viewed by a plurality of photomultiplier tubes to reconstruct an image showing distinct features of a specimen stained with different dyes. The images may also be constructed using a single detector in a time-multiplex fashion and using a computer to construct the image.

Description of the Drawings

[0019] In the drawings where like numerals refer to like components throughout the several views,
[0020] Figure 1 is a diagram showing a prior art 5 optical confocal microscope system.
[0021] Figure 2 is a diagram showing a prior art laser scanning confocal imaging system.
[0022] Figure 3 shows the response spectra of the various components of Figure 2.
[0023] Figure 4 shows a typical layout of the various components for the present invention.
[0024] Figure 5 is a diagram showing a time-multiplex single detector laser scanning confocal imaging system.
[0025] Figure 6 shows the absorption and emission spectra of various fluorochromes.
[0026] Figure 7 shows the response spectra of the various filters and dichroic reflectors used in the time-multiplex single detector laser scanning confocal imaging system of Figure 5.
[0027] Figure 8 is a diagram showing a multi-detector, multicolor optical confocal microscope system using three detectors.

Detailed Description of the Preferred Embodiment

[0028] In the following detailed description of the preferred embodiments, references made to the accompanying drawings which form a part hereof and in which is shown by way of illustration specific embodiments in which the invention may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention, and it is to be understood that other embodiments may be utilized. The following detailed description is, therefore, not to be taken in a limiting sense, and the scope of the present invention is defined by the appended claims.

[0029] Figure 4 shows a typical layout of the various components for the present invention. The layout is similar to that of the MRC-600 laser scanning confocal imaging system manufactured by BioRad Microscience Ltd. of Hemel Hempstead, Herts, England. The present laser scanning confocal microscope system is also adaptable for use with a number of upright and inverted microscopes available from microscope vendors such as Nikon, Zeiss, Olympus and Leitz. The main optics and scanner head of the present invention are housed cabinet 401. The source of laser light 403 is supported near the cabinet so that the multi-line laser light enters the cabinet. In the configuration shown, the incident laser light exits the cabinet to enter the microscope 405 from the top. The reflected of emitted light is received from the microscope along the same optical path as the incident laser light.

[0030] Cabinet 401 also contains the detectors, which in the preferred embodiments are photomultiplier tubes. Those skilled in the art will readily recognize that other detectors may be used such as CCD devices, vidicon tubes, etc. The detectors within the cabinet and the scanners are connected to computer 407 which constructs the images of the specimen and displays them on display 409.

[0031] Figure 5 shows a multi-color, single-detector scanning laser confocal imaging system of an preferred embodiment of the present invention. The multi-color images are serially constructed from a single detector by time-multiplex gathering of information by the computer. The laser selected for use in this embodiment is also an air-cooled krypton/argon ion laser, Model 5470-KBR available from ILT. Those skilled in the art will readily recognize that, by appropriate selection of filters and dichroic mirrors, other lasers, such as a krypton laser could be used with the present invention as described in conjunction with Figure 5, such as an air-cooled krypton laser, Model 5470K available from Ion Laser Technology. Other vendors produce lasers which would be acceptable in analogy with the represent invention.

[0032] An instrument comprising a laser emitting at wavelengths other than 488nm, 568nm, and 647nm would not, however, be included in the appending claims.

[0033] Krypton/argon ion laser 601 produces dominant spectral lines at 488nm, 568nm, and 647nm. Table 1 de-

scribes the selection of filters and mirrors to be used with the krypton/argon laser. In the construction of the preferred embodiment of the present invention described in conjunction with Figure 5, the excitation filter 607, the dichroic mirror 609 and the emission filter 611 are all mounted together in a single filter block 605. In this fashion, matched sets of these filters may be easily substituted without disturbing the other optics by merely replacing the filter blocks 605.

TABLE 1

Filter Block	Laser Line	Excitation Filter	Dichroic Mirror	Emission Filter
Blue	488 nm	488 DF 10	500 DCLP	522 DF 35
Yellow	568 nm	568 DF 10	585 DCLP	600 DF 20
Red	647 nm	647 DF 10	660 DCLP	665 LP

[0034] The krypton gas of the laser produces spectral lines at 476/482nm, 520nm, 568nm and 647nm, among others. The argon gas produces spectral lines at 488nm and 514nm, among others. The krypton/argon laser is designed to suppress all lines except those at 488nm, 568nm and 647nm. Since the 488nm line from the argon gas excites fluorescein more efficiently, the mixed gas krypton/argon laser is preferred. Those skilled in the art will readily recognize, however, that adequate power from the 476/482nm line of a krypton can excite fluorescein for use in analogy with the present invention.

[0035] The preferred krypton/argon laser from ILT is air-cooled since it is inexpensive and easily cooled. Since liquid or water-cooled lasers suffer much vibration due to the pumping of the liquid around the tube for coolant, air-cooled lasers are preferred. Air-cooled lasers, of course, are also preferred because of the cost differential between air-cooled and water-cooled high-powered lasers.

[0036] Referring once again to Figure 5, the specimen 603 is dyed with three dyes: fluorescein, Lissamine rhodamine (or Texas Red™), and cyanine 5.18. These dyes can be excited by the laser lines described for the krypton/argon laser and be detected by the photomultiplier tube without interference from the other dyes. In some cases, however, higher light levels for the cyanine 5.18 dye may be required due to the lower sensitivity of the photomultiplier tube to red light.

[0037] Those skilled in the art will readily recognize that a krypton laser having dominant lines at 476/482nm, 520nm, 568nm and/or 647nm may be used to excite selected dyes and obtain results similar to those described above. Thus, a krypton laser with minor adjustments made to the filters and dichroic mirrors will produce acceptable results useable in analogy with the present invention.

[0038] Figure 6 should be viewed in conjunction with an explanation of laser 601 of Figure 5. Figure 6 shows a graph of the absorption spectra and emission spectra of selected dyes which may be used to stain specimen 603 of Figure 5. The graph of Figure 6 shows the spectra curves normalized on a scale of zero to one hundred as a measure of relative intensity to one another. Curve 701 corresponds to the absorption spectrum of fluorescein and curve 703 corresponds to the emission spectrum of fluorescein. Curve 705 corresponds to the absorption spectra of Lissamine rhodamine and curve 709 corresponds to the emission spectrum of Lissamine rhodamine. Curve 711 corresponds to the absorption spectrum of cyanine 5.18 and curve 713 corresponds to the emission spectrum of cyanine 5.18. With proper selection of excitation light, the emission and absorption spectra of the various selected dyes are sufficiently removed to allow filtering and detection by separate photo multiplier tubes as shown in Figure 5.

[0039] Figure 7 is a description of the response of the various filters of Figure 5. The response curves are described as being part of one of three filter blocks: blue, yellow and red. Response curve 801 corresponds to the response curve of a 488 emission filter Part No. 488 DF10 available from Omega Instruments, Inc. Curve 803 is the dichroic mirror 500 DCLP long pass filter available from Omega. Response curve 805 corresponds to emission filter 522 DF 35 available from Omega. Response curves 801, 803 and 805 are part of the blue filter block.

[0040] For the yellow filter block, response curve 807 corresponds to the 10 nanometers wide emission filter Part No. 568 DF 10. Curve 809 corresponds to dichroic mirror Part No. 585 DCLP available from Omega. Curve 811 corresponds to the emission filter Part No. 600 DF 20 also available from Omega.

[0041] The red filter set corresponds to curves 813, 815 and 817. Curve 813 is the response curve for the excitation filter Part No. 647 DF 10. Curve 815 corresponds to the long pass dichroic mirror filter 660 DCLP available from Omega. Curve 817 corresponds to the emission filter which is a long pass filter 665 LP also available from Omega.

[0042] Referring once again to Figure 5, a time-multiplexed, three-colored confocal microscope is implemented by changing the filter blocks 605 corresponding to the colors desired to be viewed in the specimen 603. The filter block 605 comprised of excitation filter 607, the dichroic mirror 609 and the emission filter 609 are all changed together to correspond to the specific dye being viewed. The multispectral line laser light 600 from laser 601 enters the selected excitation filter 607 which narrowly selects one of the available laser lines from the krypton/argon laser. The laser light with the selected line is reflected by preselected dichroic mirror 609 which corresponds to the selected line.

[0043] Dichroic mirror 609 will reflect the laser light containing the selected line onto the XY scanning unit 613 which

causes the laser beam to be raster scanned on the specimen. XY scanning unit 613 is available within the BioRad Microscience MRC-600 confocal imaging system. The scanning unit 613 contains two mirrors which are connected to galvanometers which move the mirrors at selected scanning rates. One of the mirrors is responsible for generating an X axis scan while the other mirror produces a Y axis scan. The scanning is synchronized to the receipt of images by the photomultiplier tubes and the generation of computer images by means of a scan card and frame store in the computer 620.

[0044] The scanned laser light containing the laser line of interest is projected through the microscope objective onto specimen 603. The dye corresponding to the selected laser line fluoresces and emits a longer wavelength light in response to the excitation. The longer wavelength emitted light is passed back along the same optical path through scanning unit 613 to dichroic mirror 609. The longer wavelength emitted light passes through dichroic mirror 609 to emission filter 611. The emission filter is selected from the group described above corresponding to the selected laser line and dye to be viewed.

[0045] The emitted light through filter 611 is passed to the photomultiplier tube 615 where the scanned image is received, converted to electrical images and passed to the computer 620. The computer 620 reconstructs the image for the particular dye being excited by the selected line.

[0046] This process is duplicated for each of the colors desired to be viewed. The filter block 605 containing filters 607, 611 and dichroic mirror 609 can be easily changed without disturbing the alignment of the laser, the specimen or the photomultiplier tube. The specimen can then be scanned with the filter block for the second dye. However, by changing filter blocks, the region on the focal plane scanned may shift slightly in the XY plane due to the small differences in the angle of the dichroic mirrors in the filter blocks. After acquiring the images, the computer can combine and align the two images to produce a true two-color or pseudo color image. Quite often a pseudo color image is created wherein one color on the computer generated image corresponds to a detected color from the specimen. The color detected from the specimen may not correspond to the color used by the computer to highlight the features stained by that particular dye since it may be more aesthetically pleasing to view higher contrasting colors than the actual colors received from the specimen.

[0047] In addition, the filter block 620 may be replaced by the third filter set and a third color scanned and combined with the previous two images to generate a true three-colored image by computer 615.

[0048] Figure 8 shows a true three-color laser scanning confocal microscope which does not require the substitution of filter blocks to generate a three-color image. With the embodiment shown in Figure 8, a simultaneous three-color image may be scanned in which all three laser lines excite the dyes simultaneously. Laser 601 is a krypton/argon laser as described above. The multi-line laser light 600 passes through a broad-bandpass filter 907 where it is reflected by multi-passband dichroic mirror 909. The laser excitation light 600 is raster scanned by scanning unit 913 and enters the microscope eyepiece.

[0049] The specimen 603 is stained with dyes as described above which fluoresce when excited. The emitted light follows the same optical path followed by the excitation laser light through the scanning unit 913. The emitted light has a longer wavelength than the excitation light and passes through multi-passband dichroic mirror 909. The emitted light then strikes a second dichroic mirror 917 selected to reflect blue light and pass the other colors. Photomultiplier tube 915 receives blue light emitted by fluorescein as filtered by emission filter 607.

[0050] The longer wavelength emitted light has passes through the second dichroic mirror 917 where it strikes a third dichroic mirror 919 selected to reflect red light and pass longer wavelength red light. Photomultiplier tube 921 receives red light emitted by Lissamine rhodamine (or Texas Red™) as filtered by emission filter 925. Photomultiplier tube 923 receives longer wavelength red light emitted by cyanine 5.18.

[0051] Fluorescein conjugated probes are one of several dyes available for use in conjunction with the preferred embodiments of the present invention in staining specimens and examining selected features. Table 2 describes a number of dyes that may be used with the present invention.

TABLE 2

Fluorescent Dyes for Conjugation

- Fluorescein isothiocyanate (FITC)
- Borate-dipyrromethane (•Bodipy)
- Lucifer Yellow
- Tetramethylrhodamine isothiocyanate (TRITC)
- Lissamine rhodamine
- Texas Red™ (from Molecular Probes, Inc.)
- Allophycocyanine
- Ultralite T-680™ (Ultra Diagnostics Corp, Seattle WA)

TABLE 2 (continued)

Fluorescent Dyes for Conjugation

Ultralite T-700™ (Ultra Diagnostics Corp, Seattle WA)

Carboxycyanine derivatives

(for example, cyanine 5.18 from Molecular Probes or Jackson Immunoresearch Labs, Inc. of West Grove, PA)

Nuclear Stains

Chromomycin A3 (Sigma Chemical co.) (DNA specific and spectrally similar to fluorescein)

Ethidium bromide

Propidium iodide

LD700 (from Exciton Chemical Co., Dayton, OH)

Acridine Orange

Pararosaniline (end product of Feulgen reaction)

Physioloaical Indicators

Fluo-3™ (calcium indicators) (Molecular Probes Inc.)

Rhod-2™ (calcium indicators)

SNAFL™ (ph indicators)

SNARF™ (ph indicators)

[0052] While the present invention has been described in connection with the preferred embodiment thereof, it will be understood that many modifications will be readily apparent to those of ordinary skill in the art. The present invention is intended to be used in many fields of art analogous to, and in addition to the fields described above, including flow cytometry. Therefore, it is manifestly intended that this invention be limited only by the appending claims.

Claims

1. A multi-color confocal microscopy system for use with an optical microscope in viewing a specimen stained with a plurality of stains, which stains fluoresce when excited by light of particular light frequencies, comprising:

   a single laser means (601) for producing multi-line incident laser light having multiple excitation lines from a single laser light source at wavelengths of 488nm, 568nm and 647nm, each of said excitation lines corresponding to an excitation frequency of only one of the plurality of stains;
   means (613, 913) for directing said incident laser light into the microscope and for receiving emitted light from the microscope, said emitted light having simultaneous multiple fluorescent emissions, each of said fluorescent emissions corresponding to an excitation frequency of one of the stains and each excitation frequency of each stain corresponds to a single excitation line from the single laser;
   detector means (615, 915, 921, 923) positioned to receive said emitted light for converting said emitted light into electrical signals; and
   control means (620) connected to said detector means for accumulating said electrical signals and for producing a plurality of images of the specimen at a precise focal plane, each of images corresponding to one of said lines of said laser.

2. The multi-color confocal microscopy system according to claim 1 further including means connected to said laser means for suppressing the production of further excitation lines which would excite more than one stain.

3. The multi-color confocal microscopy system according to claim 1 further including three filter means (607) arranged to be positioned alternatingly in the path of said incident laser light for selectively passing a selected one of said excitation lines from said laser means.

4. The multi-color confocal microscopy system according to claim 1 further including filter means (611, 911, 609, 909) positioned in the path of said emitted light for selectively passing a selected emission of said fluorescent to said detector means.

5. The multi-color confocal microscopy system according to claim 4 wherein said filter means includes a dichroic

mirror (609, 909, 917, 919) positioned in the path of said emitted light for separating each of said fluorescent emissions into separate line-paths and for directing each of said line-paths into one of a plurality of detectors

6. The multi-color confocal microscopy system according to claim 3 wherein said detector means includes means for detecting one line at a time and forming a multi-color optical image on a time-multiplexed basis.

7. The multi-color confocal microscopy system according to claim 1 wherein said means for directing further includes means for scanning said incident laser light onto the specimen in a raster-scan format and wherein said detector means further includes means for descanning said emitted light.

8. The multi-color confocal microscopy system according to claim 1 wherein said control means further includes a computer having a display means for displaying the specimen in a plurality of pseudo-colors, each of said pseudo-colors corresponding to the fluorescent emission of the emitted light of one of the stains.

9. The multi-color confocal microscopy system according to claim 1 wherein said laser means includes a single krypton-argon laser.

10. The multi-color confocal microscopy system according to claim 9 wherein said krypton-argon laser is configured to produce a plurality of laser lines such that each line excites at most only one of Fluorescein. Lissamine Rhodamine, and cyanine 5.18 stains.

11. The multi-color confocal microscopy system according to claim 1 wherein said detector means further includes photomultiplier tubes.

12. A method of viewing a specimen stained with a plurality of stains, which stains fluoresce when excited by light of particular frequencies, comprising the steps of:

producing multi-line incident laser light from a single laser having multiple excitation lines from a single laser light source at wavelengths of 488nm, 568nm and 647nm, each of said excitation lines corresponding to single excitation frequency of one of the stains;
directing said incident laser light into a microscope holding the specimen;
receiving emitted light from the microscope, said emitted light having multiple fluorescent emissions, each of said fluorescent emissions corresponding to a single excitation frequency of one of the stains:
detecting said emitted light and converting said emitted light into electrical signals; and
accumulating said electrical signals and producing a plurality of images of the specimen at a precise focal plane, each of said images corresponding to one of said lines of said laser.

13. The method according to claim 12 further including the step of suppressing the production of further excitation lines which would excite more than one stain.

14. The method according to claim 12 further including the steps of filtering said incident laser light and selectively and alternatingly passing each of said excitation lines.

15. The method according to claim 12 further including the steps of filtering said emitted light and for selectively passing a selected emission of said fluorescent stain.

Patentansprüche

1. Konfokales Mehrfarben-Mikroskopiesystem zur Verwendung mit einem Lichtmikroskop beim Betrachten einer Probe, die mit mehreren Farbstoffen gefärbt ist, wobei die Farbstoffe fluoreszieren, wenn sie durch Licht mit besonderen Frequenzen angeregt werden, wobei das Mikroskopiesystem aufweist:

eine einzelne Lasereinrichtung (601) zum Erzeugen von einfallendem Mehrlinien-Laserlicht, das mehrere Anregungslinien aus einer einzelnen Laserlichtquelle bei Wellenlängen von 488nm, 568nm und 647nm aufweist, wobei jede der Anregungslinien einer Anregungsfrequenz von nur einem der mehreren Farbstoffe entspricht;
Einrichtungen (613, 913) zum Leiten des einfallenden Laserlichts in das Mikroskop und zum Empfangen von emittiertem Licht aus dem Mikroskop, wobei das emittierte Licht gleichzeitig mehrere Fluoreszenzemissionen

aufweist, jede der Fluoreszenzemissionen einer Anregungsfrequenz eines der Farbstoffe entspricht und jede Anregungsfrequenz jedes Farbstoffes einer einzelnen Anregungslinie aus dem einzelnen Laser entspricht;
Detektoreinrichtungen (615,915,921,923), die angeordnet sind, um das emittierte Licht zu empfangen und das emittierte Licht in elektrische Signale umzuwandeln; und
Steuereinrichtungen (620), die mit den Detektoreinrichtungen verbunden sind, zum Speichern der elektrischen Signale und zum Erzeugen mehrerer Bilder der Probe in einer präzisen Brennebene, wobei jedes der Bilder einer der Linien des Lasers entspricht.

2. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 1, das ferner Einrichtungen aufweist, die mit der Lasereinrichtung verbunden sind, um die Erzeugung weiterer Anregungslinien zu unterdrücken, die mehr als einen Farbstoff anregen würden.

3. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 1, das ferner drei Filtereinrichtungen (607) aufweist, die angeordnet sind, um abwechselnd im Weg des einfallenden Laserlichts positioniert zu werden und eine ausgewählte der Anregungslinien aus der Lasereinrichtung selektiv durchzulassen.

4. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 1, das ferner Filtereinrichtungen (611,911,609,909) aufweist, die im Weg des emittierten Lichts angeordnet sind, um eine ausgewählte Emission der Fluoreszenz zu den Detektoreinrichtungen selektiv durchzulassen.

5. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 4, wobei die Filtereinrichtungen einen dichroitischen Spiegel (609,909,917,919) aufweisen, der im Weg des emittierten Lichts angeordnet ist, um jede der Fluoreszenzemissionen in getrennte Linienwege aufzuteilen und jeden der Linienwege in einen von mehreren Detektoren zu leiten.

6. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 3, wobei die Detektoreinrichtungen Einrichtungen zum Detektieren jeweils einer Linie zu einer Zeit und Bilden eines optischen Mehrfarbbildes auf einer Zeitmultiplexgrundlage aufweisen.

7. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 1, wobei die Leiteinrichtungen ferner Einrichtungen zum Scannen des einfallenden Laserlichts auf der Probe in einem Rasterscannformat aufweisen und die Detektoreinrichtungen ferner Einrichtungen zum Entscannen des emittierten Lichts aufweisen.

8. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 1, wobei die Steuereinrichtungen ferner einen Computer mit Anzeigeeinrichtungen zum Anzeigen der Probe in mehreren Pseudofarben aufweisen, wobei jede der Pseudofarben der Fluoreszenzemission des emittierten Lichts eines der Farbstoffe entspricht.

9. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 1, wobei die Lasereinrichtung einen einzelnen Krypton-Argon-Laser aufweist.

10. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 9, wobei der Krypton-Argon-Laser ausgebildet ist, um mehrere Laserlinien zu erzeugen, so daß jede Linie höchstens einen der Fluorescein-, Lissamin-Rhodamin- und Cyanin-5.18-Farbstoffe anregt.

11. Konfokales Mehrfarben-Mikroskopiesystem nach Anspruch 1, wobei die Detektoreinrichtungen ferner Sekundärelektronenvervielfacher-Röhren aufweisen.

12. Verfahren zum Betrachten einer Probe, das mit mehreren Farbstoffen gefärbt ist, wobei die Farbstoffe fluoreszieren, wenn sie durch Licht bestimmter Frequenzen angeregt werden, das die Schritte aufweist:

Erzeugen von einfallendem Mehrlinien-Laserlicht aus einem einzelnen Laser, der mehrere Anregungslinien aus einer einzelnen Laserlichtquelle bei Wellenlängen von 488nm, 568nm und 647nm aufweist, wobei jede der Anregungslinien einer einzelnen Anregungsfrequenz eines der Farbstoffe entspricht;
Leiten des einfallenden Laserlichts in ein Mikroskop, das die Probe hält;
Empfangen von emittiertem Licht aus dem Mikroskop, wobei das emittierte Licht mehrere Fluoreszenzemissionen aufweist und jede der Fluoreszenzemissionen einer einzelnen Anregungsfrequenz eines der Farbstoffe entspricht;
Detektieren des emittierten Lichts und Umwandeln des emittierten Lichts in elektrische Signale; und

Akkumulieren der elektrischen Signale und Erzeugen mehrerer Bilder der Probe in einer präzisen Brennebene, wobei jedes der Bilder einer der Linien des Lasers entspricht

13. Verfahren nach Anspruch 12, das ferner den Schritt zum Unterdrücken der Erzeugung weiterer Anregungslinien aufweist, die mehr als einen Farbstoff anregen würden.

14. Verfahren nach Anspruch 12, das ferner die Schritte zum Filtern des einfallenden Laserlichts und zum selektiven und abwechselnden Durchlassen jeder der Anregungslinien aufweist.

15. Verfahren nach Anspruch 12, das ferner die Schritte zum Filtern des emittierten Lichts und zum selektiven Durchlassen einer ausgewählten Emission des Fluoreszenzfarbstoffes aufweist.

Revendications

1. Système de microscope polychrome à foyer commun pour une utilisation dans un microscope optique lors de l'observation d'un échantillon maculé d'une pluralité de teintes, teintes devenant fluorescentes lors d'une excitation par la lumière de fréquences lumineuses particulières, comprenant :

   - un moyen unique de laser (601) pour produire une lumière incidente de laser à plusieurs lignes présentant plusieurs lignes d'excitation à partir d'une seule source de lumière laser à des longueurs d'onde de 488 nm, 568 nm et 647 nm, chacune desdites lignes d'excitation correspondant à une fréquence d'excitation d'une seule teinte de la pluralité de teintes;

   - un moyen (613, 913) pour diriger ladite lumière laser incidente dans le microscope et pour recevoir une lumière émise par le microscope, ladite lumière émise possédant plusieurs émissions fluorescentes simultanées, chacune desdites émissions fluorescentes correspondant à une fréquence d'excitation d'une des teintes et chaque fréquence d'excitation de chaque teinte correspondant à une seule ligne d'excitation à partir du laser unique;

   - des moyens de détecteur (615, 915, 921, 923) positionnés afin de recevoir ladite lumière émise pour convertir ladite lumière émise en signaux électriques; et

   - un moyen de commande (620) connecté auxdits moyens de détecteur pour accumuler lesdits signaux électriques et pour produire une pluralité d'images de l'échantillon sur un plan focal précis, chacune des images correspondant à une desdites lignes dudit laser.

2. Système de microscope polychrome à foyer commun selon la revendication 1, comprenant, de plus, un moyen connecté audit moyen de laser pour supprimer la production de lignes supplémentaires d'excitation qui exciteraient plus d'une teinte.

3. Système de microscope polychrome à foyer commun selon la revendication 1, comprenant, de plus, trois moyens de filtre (607) prévus pour être positionnés, de façon alternée, sur le trajet de ladite lumière laser incidente afin de laisser passer, de façon sélective, une ligne sélectionnée desdites lignes d'excitation à partir dudit moyen de laser.

4. Système de microscope polychrome à foyer commun selon la revendication 1, comprenant, de plus, des moyens de filtre (611, 911, 609, 909) positionnés sur le trajet de ladite lumière émise pour faire passer, de façon sélective, une émission sélectionnée de ladite fluorescence vers lesdits moyens de détecteur.

5. Système de microscope polychrome à foyer commun selon la revendication 4, dans lequel ledit moyen de filtre comprend un miroir dichroïque (609, 909, 917, 919) positionné sur le trajet de ladite lumière émise afin de séparer chacune desdites émissions fluorescentes en trajets linéaires séparés et afin de diriger chacun desdits trajets linéaires dans un détecteur d'une pluralité de détecteurs.

6. Système de microscope polychrome à foyer commun selon la revendication 3, dans lequel ledit moyen de détecteur comprend un moyen pour détecter une ligne à la fois et pour former une image optique polychrome sur une base de multiplexage dans le temps.

7. Système de microscope polychrome à foyer commun selon la revendication 1, dans lequel ledit moyen de direction comprend, de plus, un moyen pour balayer ladite lumière laser incidente sur l'échantillon selon un format à balayage de trame et dans lequel ledit moyen de détecteur comprend, de plus, un moyen pour annuler le balayage de ladite lumière émise

8. Système de microscope polychrome à foyer commun selon la revendication 1, dans lequel ledit moyen de commande comprend, de plus, un ordinateur possédant un moyen d'affichage pour afficher l'échantillon selon une pluralité de pseudocouleurs, chacune desdites pseudocouleurs correspondant à l'émission par fluorescence de la lumière émise par une des teintes.

9. Système de microscope polychrome à foyer commun selon la revendication 1, dans lequel ledit moyen de laser comprend un laser unique à krypton/argon.

10. Système de microscope polychrome à foyer commun selon la revendication 9, dans lequel ledit laser à krypton/argon est configuré pour produire une pluralité de lignes laser de telle façon que chaque ligne excite au plus une seule des teintes parmi la fluorescéine, de rhodamine lissamine et de cyanine 5.18.

11. Système de microscope polychrome à foyer commun selon la revendication 1, dans lequel ledit moyen de détecteur comprend, de plus, des tubes à photomultiplication.

12. Procédé pour la visualisation d'un échantillon maculé d'une pluralité de teintes, teintes devenant fluorescentes lors d'une excitation par une lumière de fréquences particulières, comprenant les étapes suivantes :

- la production d'une lumière laser incidente à plusieurs lignes à partir d'un seul laser possédant plusieurs lignes d'excitation à partir d'une source unique de lumière laser à des longueurs d'onde de 488 nm, 568 nm et 647 nm, chacune desdites lignes d'excitation correspondant à une seule fréquence d'excitation d'une des teintes;

- la direction de ladite lumière laser incidente dans un microscope portant l'échantillon;

- la réception de la lumière émise par le microscope, ladite lumière émise possédant plusieurs émissions fluorescentes, chacune desdites émissions fluorescentes correspondant à une seule fréquence d'excitation d'une des teintes;

- la détection de ladite lumière émise et la conversion de ladite lumière émise en signaux électriques; et

- l'accumulation desdits signaux électriques et la production d'une pluralité d'images de l'échantillon sur un plan focal précis, chacune desdites images correspondant à une desdites lignes dudit laser.

13. Procédé selon la revendication 12, comprenant, de plus, une étape de suppression de la production de lignes supplémentaires d'excitation qui exciteraient plus d'une teinte.

14. Procédé selon la revendication 12, comprenant, de plus, des étapes de filtrage de ladite lumière laser incidente et de passage, de façon sélective et alternée, de chacune desdites lignes d'excitation.

15. Procédé selon la revendication 12, comprenant, de plus, des étapes de filtrage de ladite lumière émise et de passage sélectif d'une émission sélectionnée de ladite teinte fluorescente.

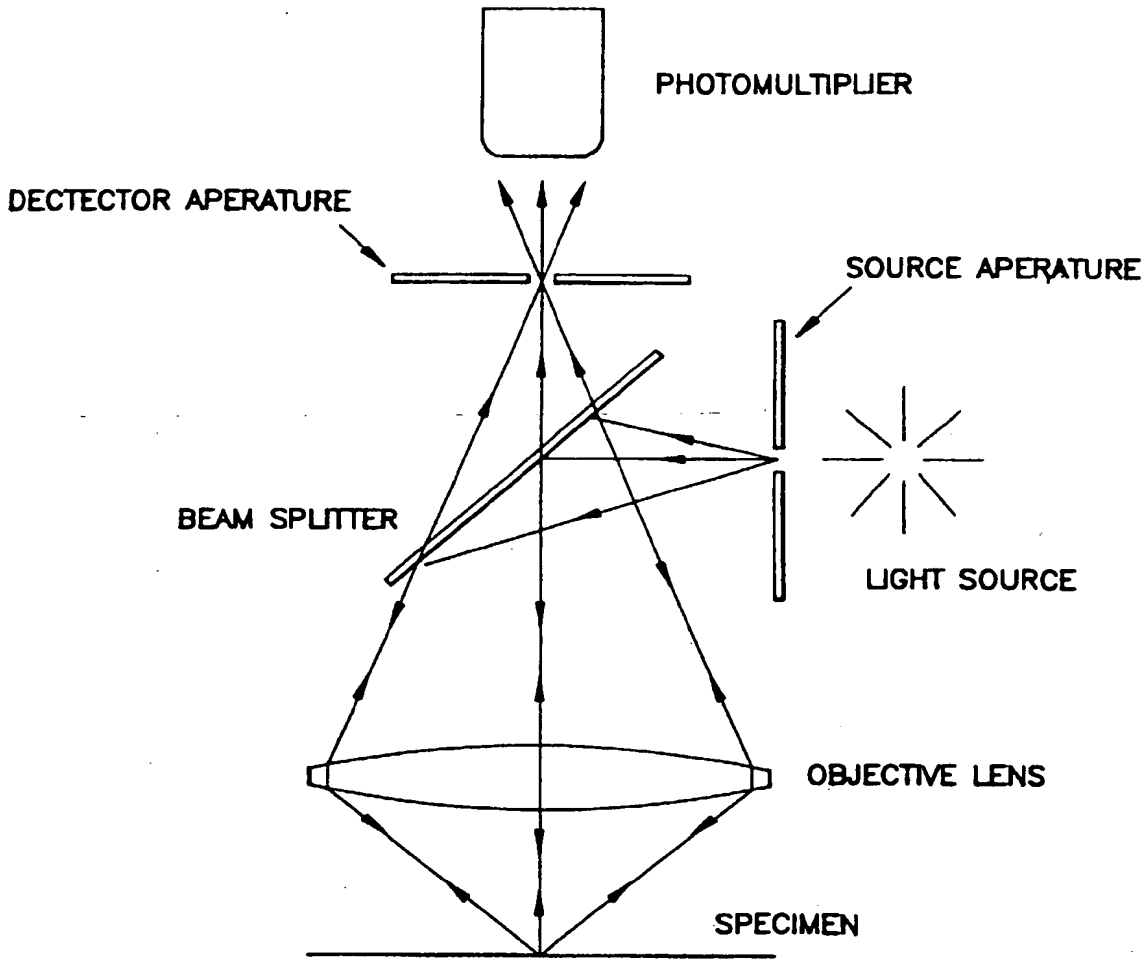


FIG. 1
PRIOR ART

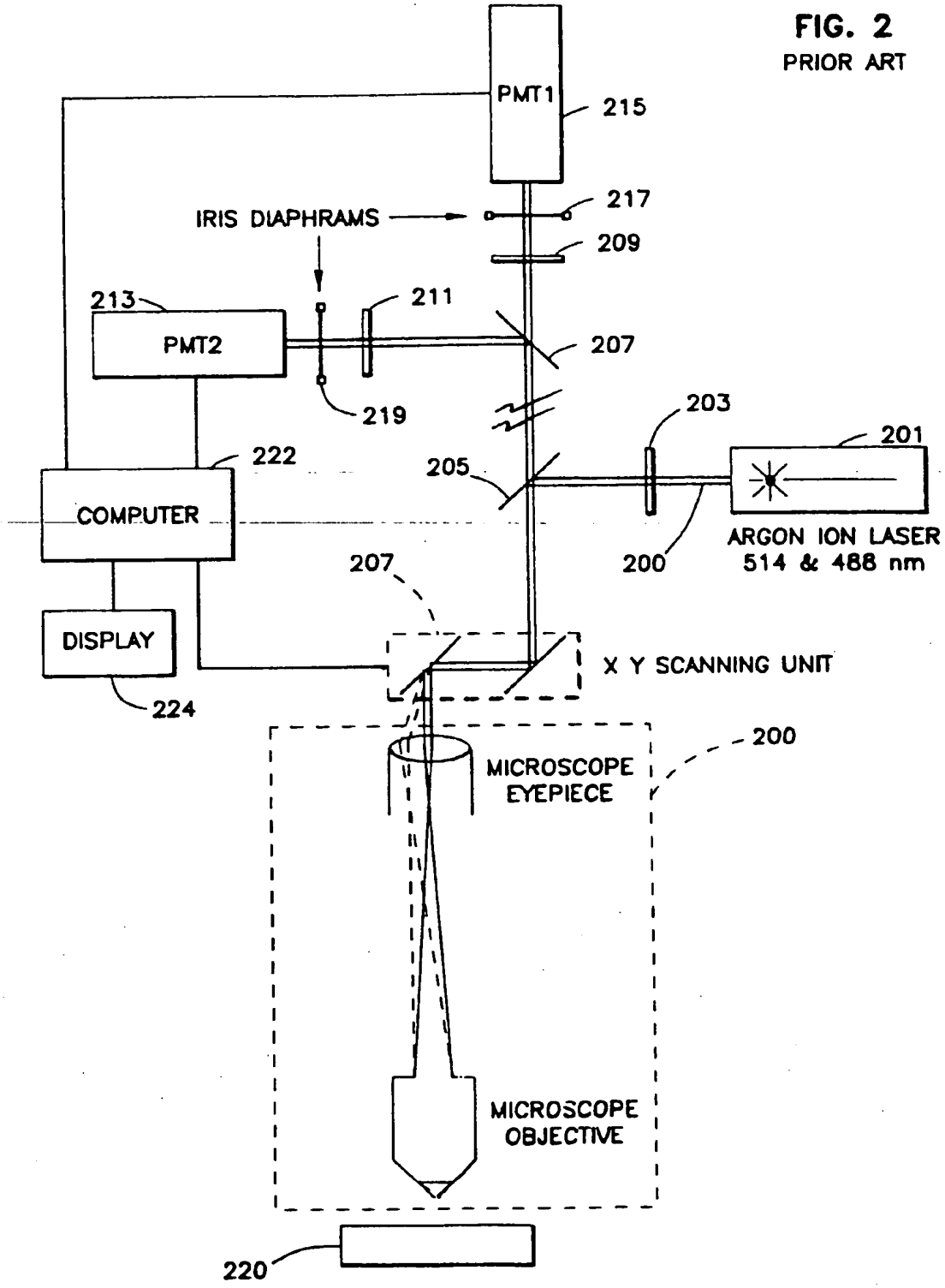


FIG. 2
PRIOR ART

FIG. 3

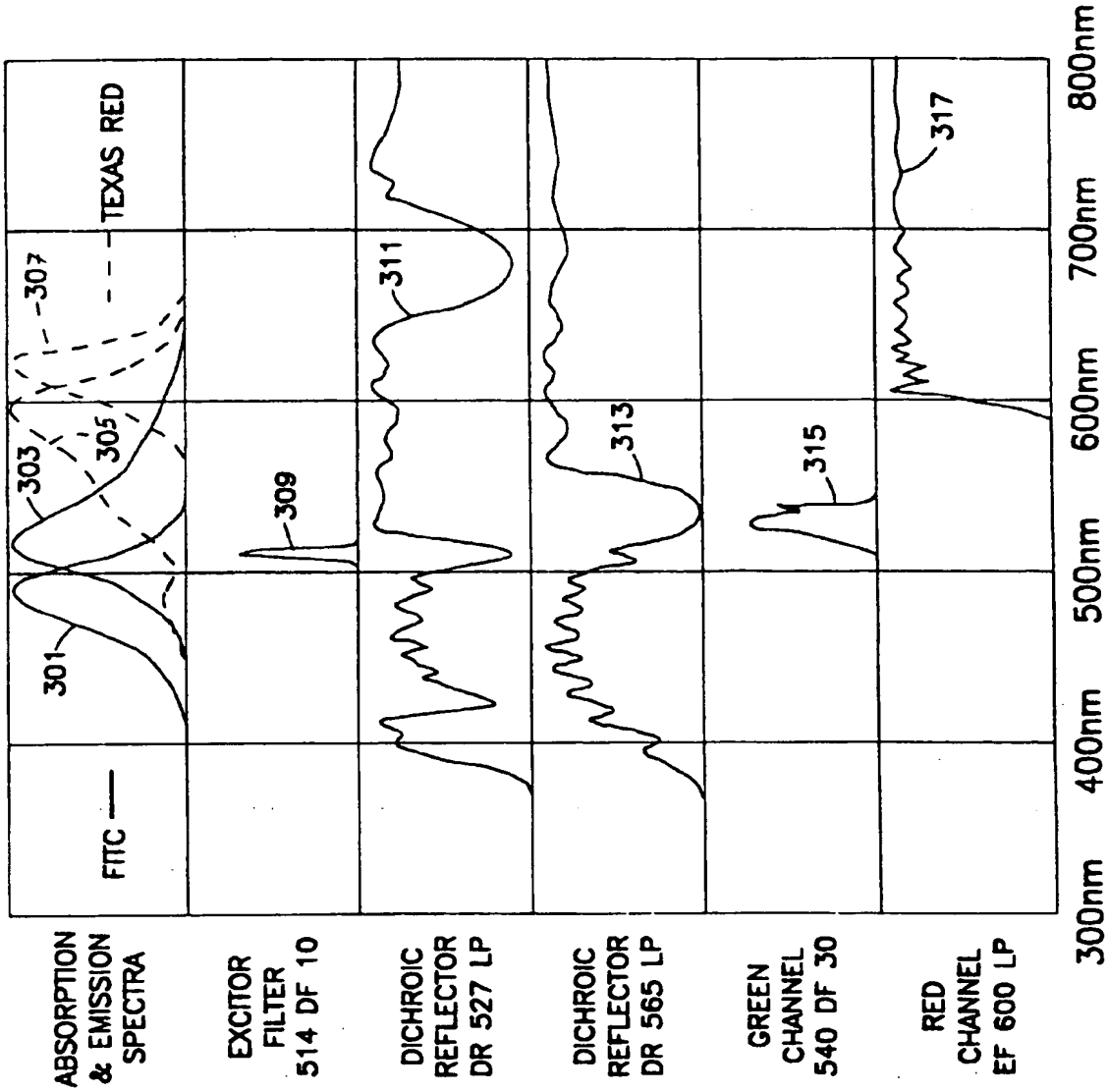

ABSORPTION & EMISSION SPECTRA
FITC
TEXAS RED
301
303
305
307
EXCITOR FILTER 514 DF 10
309
DICHROIC REFLECTOR DR 527 LP
311
DICHROIC REFLECTOR DR 565 LP
313
GREEN CHANNEL 540 DF 30
315
RED CHANNEL EF 600 LP
317
300nm
400nm
500nm
600nm
700nm
800nm

FIG. 4

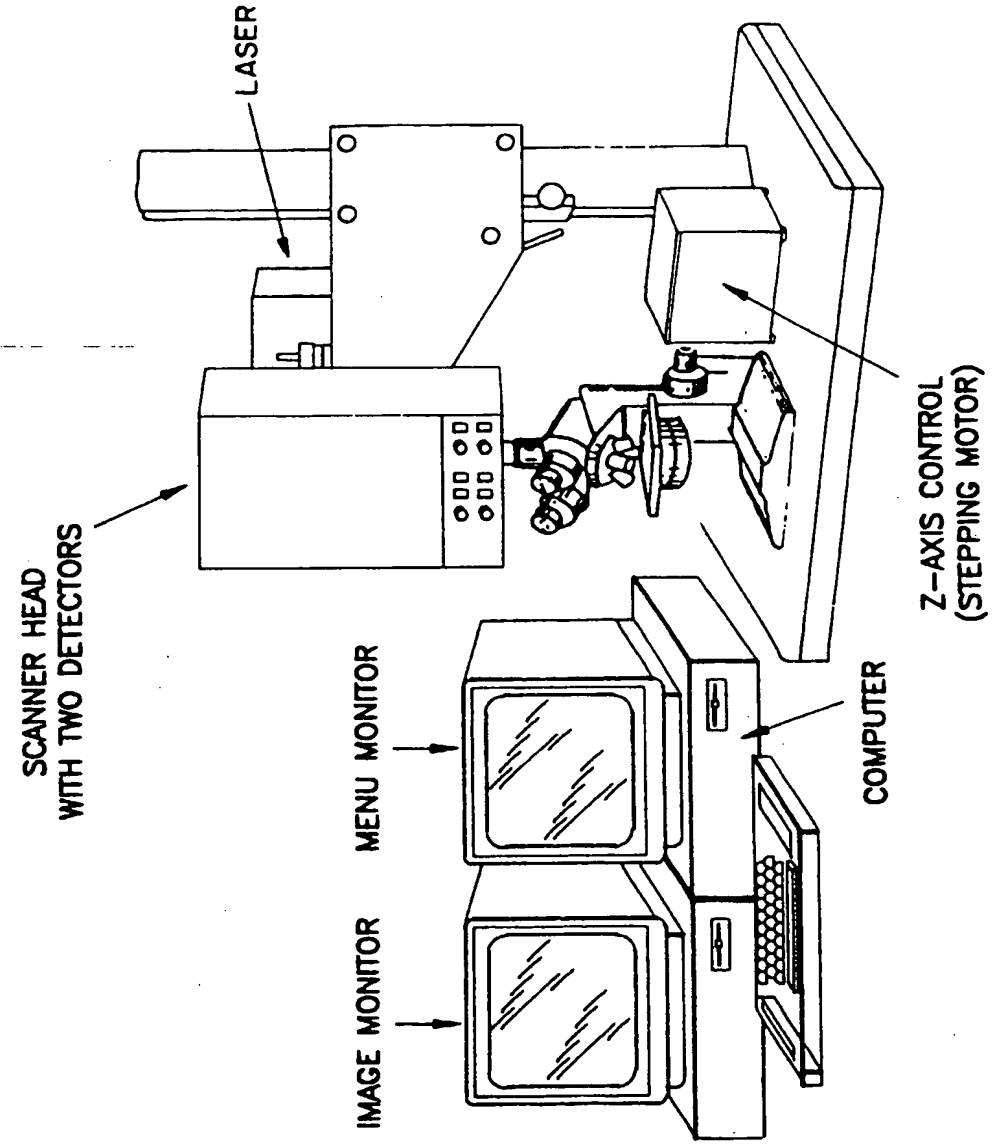
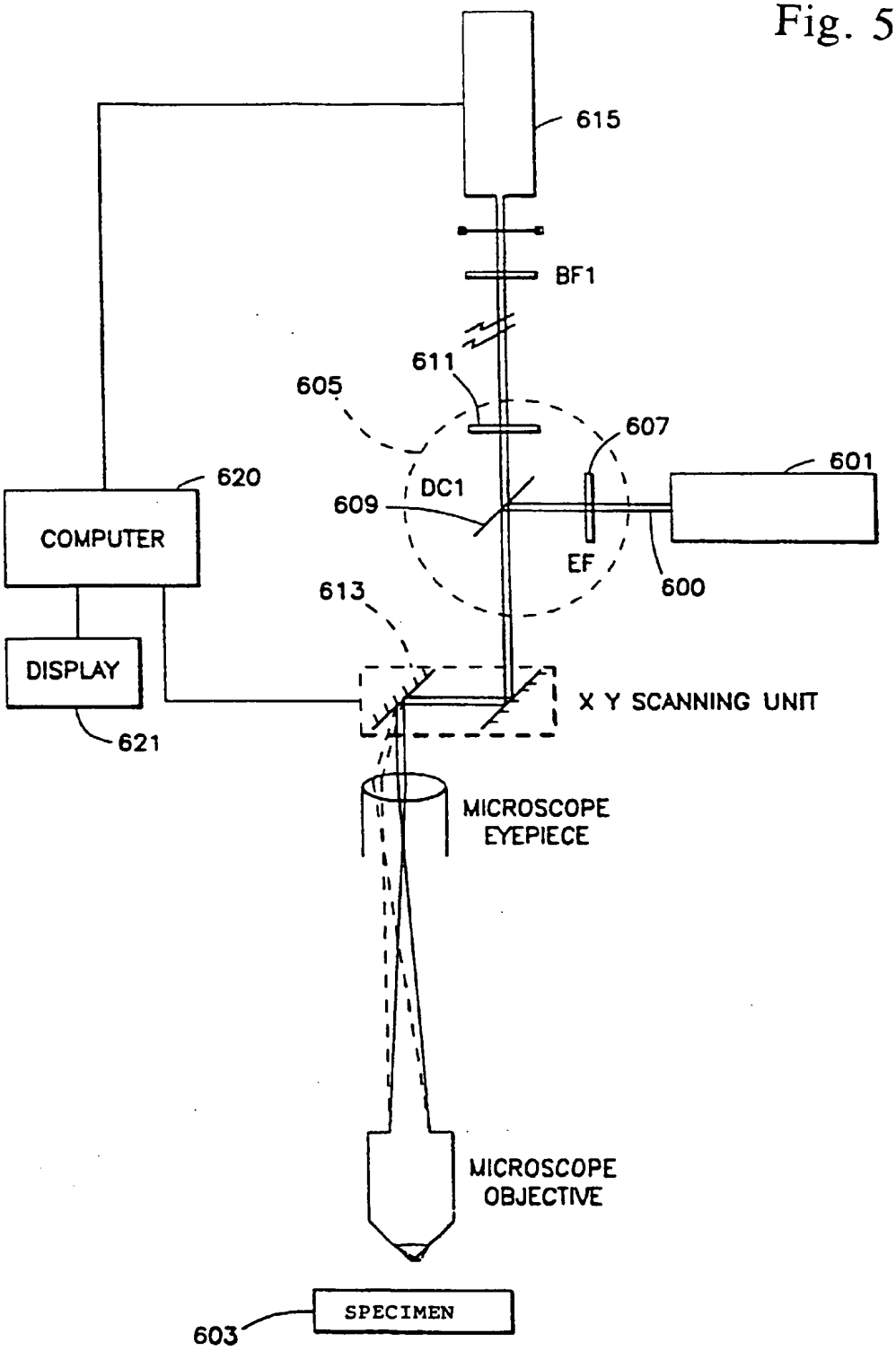

LASER
SCANNER HEAD
WITH TWO DETECTORS
MENU MONITOR
IMAGE MONITOR
COMPUTER
Z-AXIS CONTROL
(STEPPING MOTOR)

Fig. 5


615
BF1
611
605
607
601
620
DC1
609
COMPUTER
EF
600
613
DISPLAY
X Y SCANNING UNIT
621
MICROSCOPE
EYEPIECE
MICROSCOPE
OBJECTIVE
SPECIMEN
603

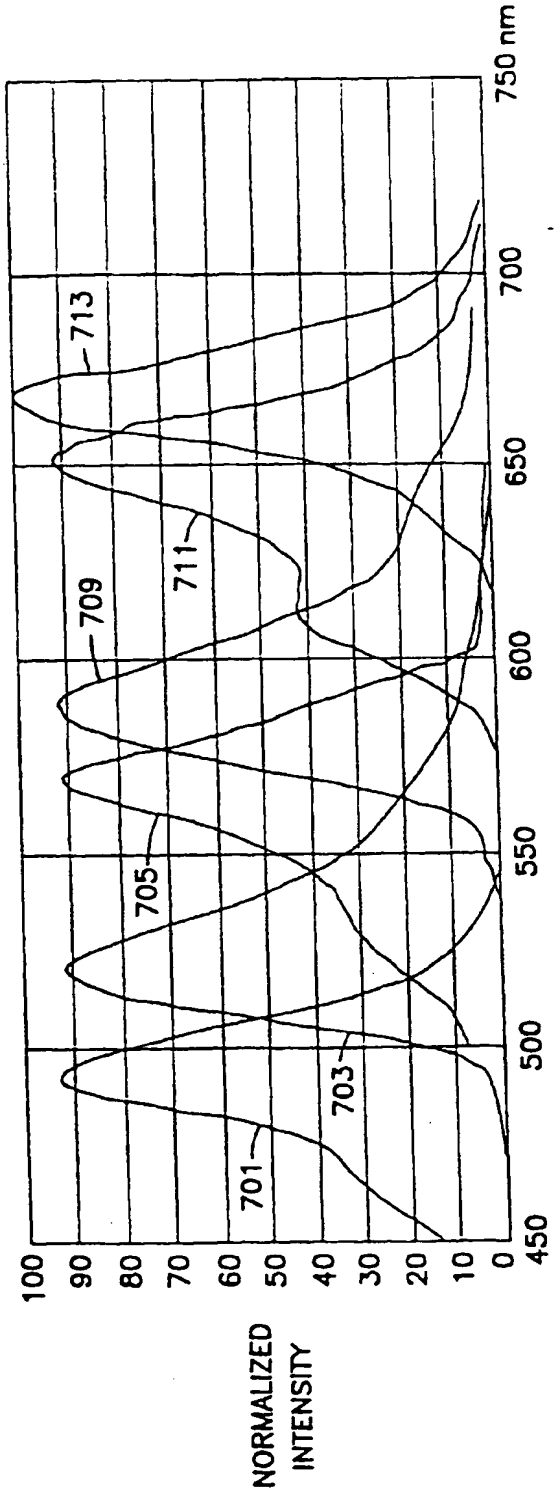

NORMALIZED INTENSITY
100
90
80
70
60
50
40
30
20
10
0
450
500
550
600
650
700
750 nm
701
703
705
709
711
713

Fig. 6

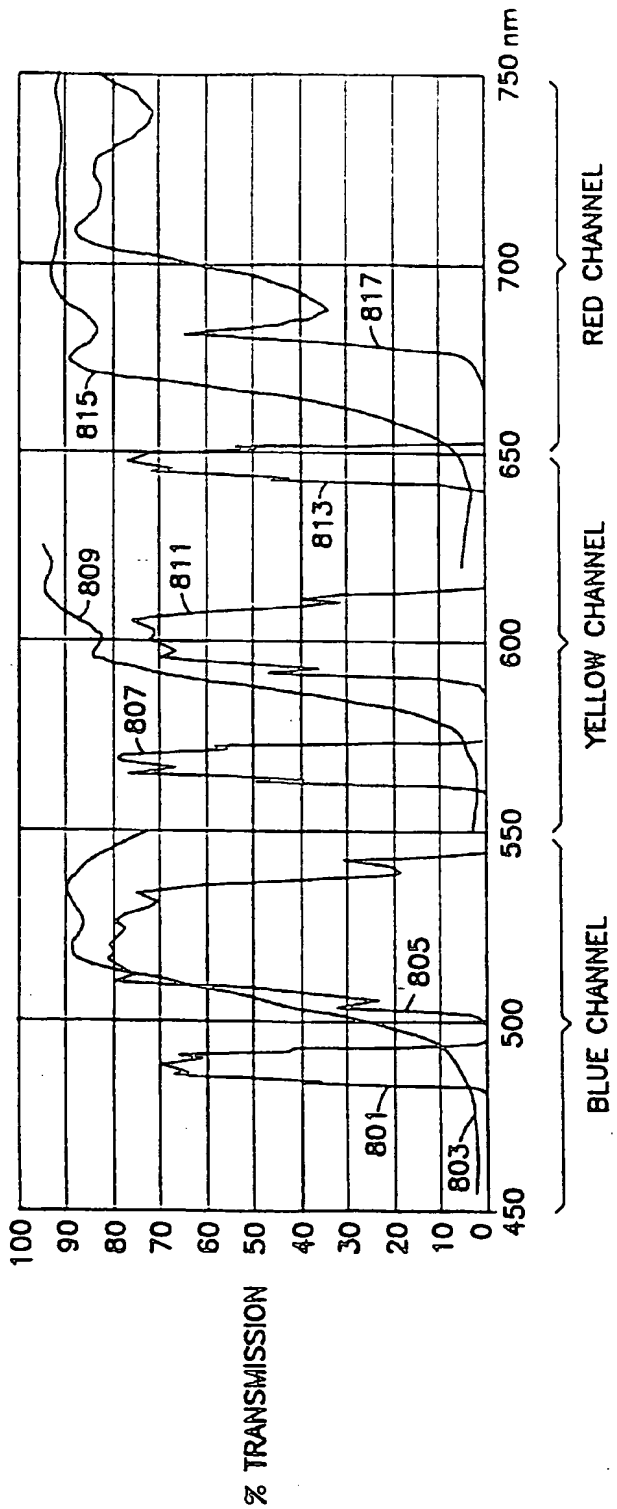
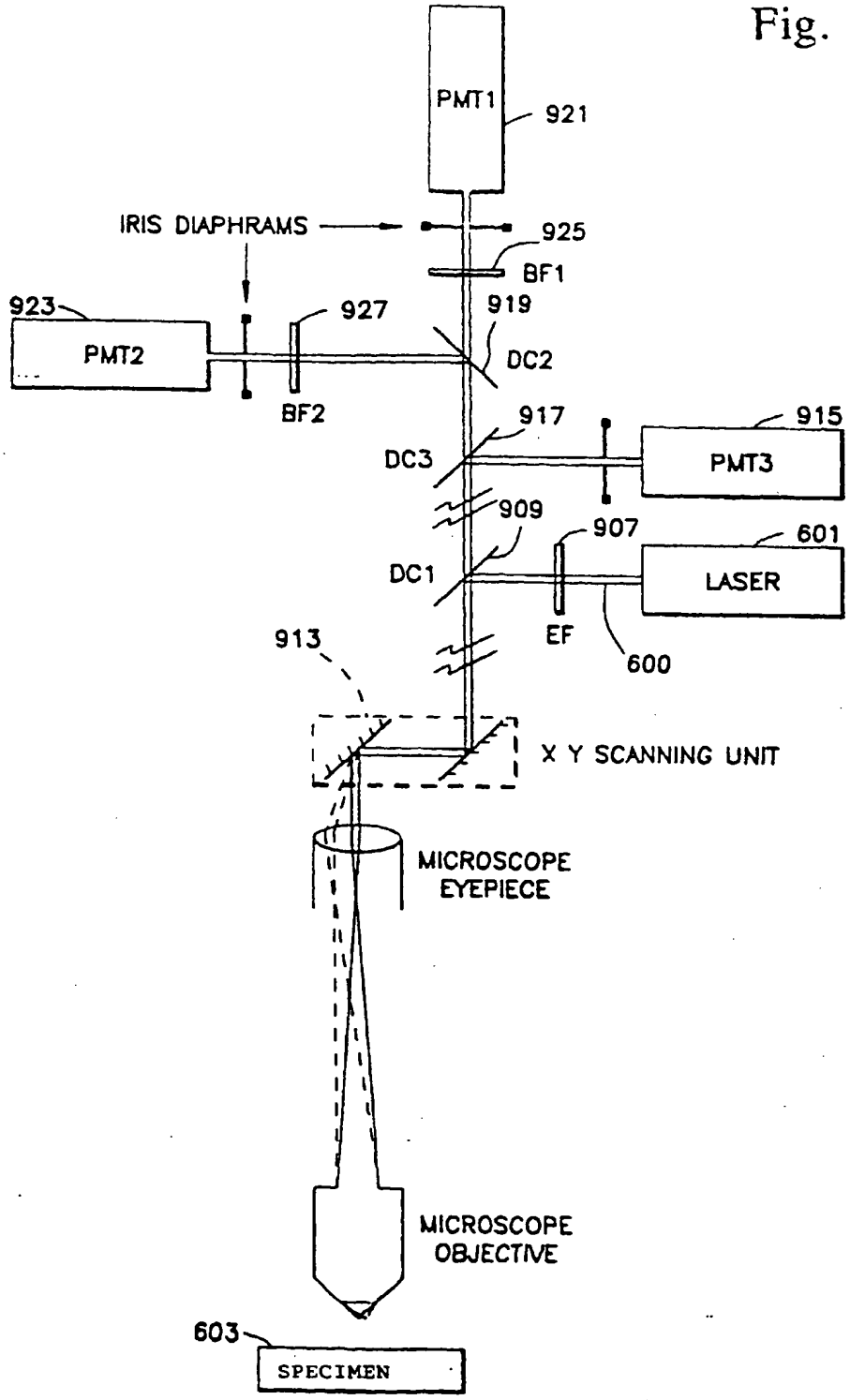

% TRANSMISSION
100
90
80
70
60
50
40
30
20
10
0
450
500
550
600
650
700
750 nm
BLUE CHANNEL
YELLOW CHANNEL
RED CHANNEL
801
803
805
807
809
811
813
815
817

Fig. 7

Fig. 8


PMT1
921
IRIS DIAPHRAMS
925
BF1
923
927
919
PMT2
DC2
BF2
917
915
DC3
PMT3
909
907
601
DC1
LASER
EF
600
913
X Y SCANNING UNIT
MICROSCOPE EYEPIECE
MICROSCOPE OBJECTIVE
603
SPECIMEN